Quality aspects of long-term stored samples

Studies in the Janus Serum Bank of Norway

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Do not go where the path may lead, go instead where there is no path and leave a trail

- Ralph Waldo Emerson

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1. Acknowledgement

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2. Abbreviations

ALT	Alanine amino transferase
ANOVA	Analysis of variance
AST	Asparagin amino transferase
BMI	Body mass index
EDTA	Ethylenediaminetetraacetic acid
CEA	Carcinoembryonic antigen
CK	Creatinine kinase
CRN	Cancer Registry of Norway
CV	Coefficient of varianc
DDT	Dichloro-diphenyl-trichloroethane
FSH	Follicle stimulating hormone
GC-MS	Gas chromatography followed by mass spectrometry
GFR	Glomerula filtration rate
HDL-chol	Heavy density lipid cholesterol
hmTHF	4-alpha-hydroxy-5-methyltetrahydrofolate
Ig	Immunoglobulin
IMMUN	Chemiluminescence immunoassay
LC-MSMS	Liquid chromatography followed by tandem mass spectrometry
LDL-chol	Light density lipid cholesterol
LH	Luteinizing hormone
MMA	Methyl malonic acid
5mTHF	5-methyltetrahydrofolate
pABG	p-aminobenzoylglutamate
PCB	Polychlorinated biphenyls
PID	Personal identification number
RBC	Red blood cell
SD	Standard deviation
SHBG	Sex Hormone Binding Globulin
SOP	Standard operation procedure
WBC	White blood cell

3. List of papers

I Randi E. Gislefoss, Tom K. Grimsrud and Lars Mørkrid. Long-term stability of serum components in the Janus Serum Bank. *Scand J Clin Lab Invest* 2008; 68(5):402-409.

II Randi E. Gislefoss, Tom K. Grimsrud and Lars Mørkrid. Stability of selected Serum proteins after long-term storage in the Janus Serum Bank. *Clin Chem Lab Med* 2009;47 (5):596-603

III Rita Hannisdal, Randi E. Gislefoss, Tom K. Grimsrud, Steinar Hustad, Lars Mørkrid, and Per Magne Ueland. Analytical Recovery of Degraded Folate in Archival Serum Samples. (Epub, *Journal of nutrition)*

IV Randi E Gislefoss, Tom K Grimsrud, Kari Høie and Lars Mørkrid. Stability of testosterone level in archival serum samples – methodological approaches, and changes in the background level. (Manuscript submitted for publication)

4. Background and aims

The Janus Serum Bank of Norway was established in 1973, and was dedicated to cancer research. It has been widely used to assess exposure and risk factors for development of cancer. The bank holds approximately 450.000 samples from more than 315.000 people that have been followed for development of cancer in the Cancer Registry of Norway, some of them for up to three decades. Today there are more than 48.000 (per December 2008) incidents of invasive cancers in the bank. Biorepositories have become an important resource in cancer epidemiology. The scientific value of an epidemiological study may be significantly enhanced by analysis of putative cancer markers in biological specimens collected from the study population. Biomarkers can be classified as markers of exposure, disease or susceptibility. Quantifying a biomarker may give more reliable and valid information on exposure than records or self reported data. The value of a study based on archival serum samples may be threatened if there are variations in preanalytical sample handling, degradation, molecular modification or loss of water during storage. One of the most important aspects of biobank activities is to evaluate the quality of the samples. The knowledge of component stability is essential for the interpretation of a study based on historical samples.

The use of archival samples generates some important question: Do we measure the most informative component? Are samples comparable when storage time differs with decades? Those and similar questions may be of high importance in the planning and interpretation of a study on archival serum samples. The overall aim of the thesis was to describe the quality of archival serum samples and discuss their usefulness in epidemiological studies.

Specific aims of the papers:

- To evaluate the impact of preanalytical sample handling, short- and long-term storage, and describe changes in selected biochemical serum components.
- To investigate long-term stability of protein biomarkers.
- To estimate recovery of folate in long-term stored serum samples.
- To measure testosterone in archival serum samples and discuss sample stability, measuring methods and changes in the background population.

5. Introduction

Epidemiology is a fundamental medical discipline that focuses on the distribution and determinants of disease in a human population. Classic epidemiologic studies use observations, register records, reports, and interviews to assess risk factors of a disease. Studies based on biomarkers of exposure, effect or susceptibility may greatly improve our understanding of host factors and environmental influence. Analysis of biochemical samples can also be used to control for confounding factors, or to evaluate the effect of bias.

Cancer epidemiology may focus on causes and precursors of the illness. Cancer is a disease that often occurs late in life and may take years to develop. The use of archival serum samples can offer a great opportunity to investigate the relationship between exposure and disease or detect early signs in the carcinogenic process.

A biobank is defined as a systematic collection of human biological samples, prepared and stored for later use and commonly linked to demographical and clinical data. Samples of body fluids or tissues are the most common material.

The potential value of a biobank depends on the quality of the samples, *i.e.* to what extent they may reflect the biological, or biochemical situation in the individual at the time of sample collection. Variation in sampling and storage conditions (*i.e.* temperature, environmental humidity, light exposure, and type of vials and caps) may be crucial for the sample quality. Both component and even the analytical methods may exhibit diverse robustness against such variations and therefore component instability has the potential to invalidate research results.

5.1 Serum

Circulating blood consists of blood cells and blood plasma comprising approximately 45% and 55%, respectively. Plasma consists of about 90% water, where many components are kept in solution: proteins (*i.e.* immunoglobulins, transport proteins, enzymes), lipids, hormones, vitamins, minerals, salts and ions, amino acids, water soluble components, inorganic electrolytes, sugar, carbon dioxide and oxygen. Plasma is separated from a blood sample to which an anticoagulant (EDTA, heparin or citrate) is added. Serum is obtained after coagulation, and in contrast to plasma does not contain fibrinogen. A blood sample in a glass tube will clot after ½-1 hour if no anticoagulant has been added.

Erythrocytes and leucocytes are trapped in the clot separating them from serum. Figure 1. shows the difference between plasma and serum.

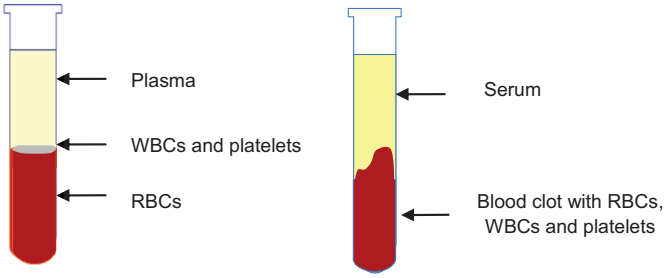

Figure1. Illustration of the components in plasma and serum

5.2 The Janus Serum Bank

The Janus Serum Bank was established in 1973 by the pathologist Olav Torgersen (1907-1978) with funding from the Norwegian Cancer Society. The biobank was dedicated to cancer research and was named after the two-faced Roman God, Janus, to symbolize the possibility for combined prospective and retrospective studies of disease. The idea was to store serum from healthy persons for identification of early markers in those who later were found to developed cancer. The Janus Serum Bank was made possible by a huge voluntary collaborative effort in Norway. The funding gave room only for simple practical and logistics solutions with respect to temperature monitoring, sample access etc. Despite these limitations and a suboptimal temperature of –25 ºC, the bank has been used extensively. The Janus Serum Bank was launched at a time when hardly anyone fully understood the value of creating such a type of specimen collection. The biobank contains serum samples from the early 1970s onwards, from a time when the diet had higher fatty content, when anti-tobacco work was in its early infancy, and environmental contaminants like PCB and DDT had not yet been banned. The bank is population-based and covers 17 of the 19 counties in Norway, not including Hordaland and Buskerud. Some 90% of the samples came from people in their forties undergoing health surveys of cardiovascular disease risk. The rest have been collected from the Red Cross Blood Bank donors in and

around Oslo, many of whom have given samples to the bank repeatedly (1). The collection of samples was terminated in 2004 except for previous Janus donors, hospitalized with a cancer diagnosis at The Radium Hospital in Oslo. The donors in the health examination cohort were largely 39–45 years old at retrieval while the blood donors were 18–65 years old. During 36 years of sample collection, the serum bank reached approximately 450.000 samples in 1 to 5 aliquots from more than 315.000 donors (per 2009). Apart from the high numbers of sera with a follow-up time of up to 35 years, the probably greatest benefits of the Janus Serum Bank are the possibility of linkage to a national high-quality cancer registry, a public registry for vital status and a public health institute for health information. The bank is one of few in a world scale referring to age, follow-up time and number of cancer cases. The donors in the Janus Serum Bank have given informed consent for the use of their samples in cancer research, mandatory by laws and regulation of personal secrecy according to requirements at the time of blood draw.

5.3 The sample collections and routines

5.3.1. Health examination samples

The first health examinations were carried out in Oslo in the period 1972–73 and included 17.400 men. In the period 1974–78 health examinations were performed in Finnmark, Sogn og Fjordane, and Oppland and comprised 54.800 men and women. In the period 1980–91 the health examinations among people in their 40s were performed across the country, and samples from about 238.000 individuals were added to the bank. The examinations were all subjected to cardiovascular disease and risk factors. In 2002 an examination on health and life condition in Troms was launched. The Janus Serum Bank received samples from 4.900 individuals from this survey.

During the enrolment in 1974–78, all samples were collected in Vacutainer™ 10 ml tubes containing 5 mg of sodium iodoacetate as a preservative for glucose (2). From 1978 till 1986, the samples were collected in plain tubes without additives, while the samples from the later years were collected on gel vials. After coagulation in room temperature and centrifugation, the serum was either transferred to new vials (glass or poly propylene tubes) before shipment, or transported on gel tubes to Ullevål Hospital in Oslo. The samples were sent, in batches of 200–300 in specially constructed cooling containers securing a temperature between +1 ºC and +10 ºC, and reached the laboratory 45–60 hours

after collection (2). The samples were analysed for cholesterol and triglycerides. The Janus Serum Bank received rest volume (0.5–2.0 ml) after the analyses. Depending on transport distance to Oslo, the analysing and subsequently freezing of the serum samples for the Janus Serum Bank could be delayed for days. Blood sampling was done at any time of the day between 8 am and 6 pm when the subject showed up without requirements of fasting. A majority of women appeared early in day-time while men mainly arrived in the afternoon.

5.3.2 Red Cross blood donor samples

The sampling routine of blood donors has followed standard routines during the years. Samples were collected at daytime between 8 am and 6 pm. The donors were non-fasting and blood was drawn with the donor in a supine position. The tubes carried no additives or separating gel and were coagulated in room temperature and stored at +4 °C over night before transport to the Janus Serum Bank. The clotting time could vary between14 – 28 hours. After centrifugation the serum was transferred to new vials before freezing. The serum was stored in 5 ml polypropylene tubes. Samples collected in 1973–75 (about 7000 vials) were lyophilized and have to be rehydrated before use. In the time period 1973–91, a blood donor gave a sample of 50 ml for the Janus Serum Bank. In the period 1997–2005 the volume was reduced to 20 ml.

5.3.3 Post diagnostic samples

Today a Janus donor, who is hospitalized at the Radium Hospital, will by admission be asked to give a sample to the Janus Serum Bank before treatment is started. The samples are collected in tubes without additives. After coagulation in room temperature and centrifugation the serum is transferred to polypropylene tubes and frozen.
All samples in the Janus Serum Bank have been stored in paper boxes covered with lids in a commercial freezer department. Exposure to light has only occurred during collecting, retrieval, thawing, and aliqouting of samples.

5.4 The Cancer Registry and linkages to Janus

In 2004, the Janus Serum Bank was transferred from The Norwegian Cancer Society to the Cancer Registry of Norway (CRN). CRN was founded in 1951 and has registered virtually all new cancer cases in Norway since 1953. Reporting cancer cases is mandatory, and the information comes from several independent sources, like pathology laboratories, hospitals, the national patients discharge registry and causes of death registry, thus securing a high grade of completeness. The quality of CRN has recently been evaluated as high (3-5). For administrative purposes, personal identification numbers (PID) have been assigned to all Norwegian residents alive in 1960 or born later. All registration of cancer is linked to the PID. The samples in the Janus Serum Bank are either labelled with the PID or a registration number that are linked to the PID. The PID ensures the possibility of identifying new cancer cases in the Janus Serum Bank. The Janus cohort is regularly linked to the cancer registry for update with incident cases. Presently, the cohort contains more than 48.000 cases of invasive cancer (per December 2009). The Cancer Registry holds the licence and has been the data handler for the Janus Serum Bank from the start. One of the important research goals of the cancer Registry is to set up testable biologically-founded hypotheses, using biological markers of exposures, and collaboration with researchers in the relevant fields. Specifically this will include utilisation of Janus Serum Bank (6).The linkage between registries with reliable data, especially concerning cancer incidence, mortality, health, lifestyle, and biological samples from a considerable number of individuals offer an extremely large study base for research. A paper on quality aspects in studies linking cancer registries and biobanks with experiences from Janus and other Nordic biobanks has recently been published (7). Linkages between the Janus Serum Bank and CRN have been used for more than 65 publications (8) from a number of research groups. The Janus Serum Bank has participated in several international collaborative studies based on linkages of large population-based biobanks and cancer registries (9). Nordic Biological Specimen Banks working group on Cancer Causes & Control (NBSBCCC) is a network consisting of 16 biobanks and 5 cancer registries. Cancer Control using Population-based Registries and Biobanks (CCPRB) is a network project within the sixth framework programme of the European Union involving 20 biobanks and 7 cancer registries located in 9 European countries. The intention of the networks has been to provide biological and statistical resources for etiological studies of

cancer, focusing on longitudinal studies and large-scale research for genetic and infectious causes of cancer. The present thesis has been performed within this network collaboration.

5.5 Sample stability

Stability can be described as the tendency of a component of a system to withstand changes and retain the initial property of a measured analyte for a period of time when the sample is stored under defined conditions (10). The aim of a clinical laboratory test is to measure the concentration or activity of a constituent in a body fluid or tissue in order to give information relevant to the clinical state of a patient. To give a correct or valid result, the composition of the samples for analysis should not change during the preanalytical phase, or at least one should have some information about the change (11). The primary sample, *i.e.* full blood, usually remains for short time (hours) at room temperature (20 to 25 °C) or at +4 °C while the secondary sample, *i.e.* serum/plasma, is kept between +4 °C and –196 °C for days, months or years. In the Janus Serum Bank, the primary samples have been kept at +4 °C for up to 28 hours and the secondary samples have been stored at –25 °C, some of them more than three decades.

The degree of stability of a blood constituent during the preanalytical phase can be influenced by a number of factors. As time and temperature have a major influence, one could determine a maximum permissible storage time with defined conditions, under which the quality may be satisfactory. The degree of stability can be described by the absolute difference, or by the ratio (percentage deviation) of results achieved from measurement at time zero and after a given period of time.

5.6 Factors affecting the outcome of component measurement

Serum components show biological variation, and several factors in the preanalytical, analytical and post-analytical phase may lead to additional variations in test results. Most measurements errors are due to preanalytical factors, while a high error rate has also been found in the postanalytical phase (12). In a study of types and frequency of mistakes in a laboratory, Plebani et al. (13) reported the following distribution of the source of mistakes: 68% preanalytical, 13% analytical and 19% postanalytical. Figure 2 presents factors that are affecting the different phases.

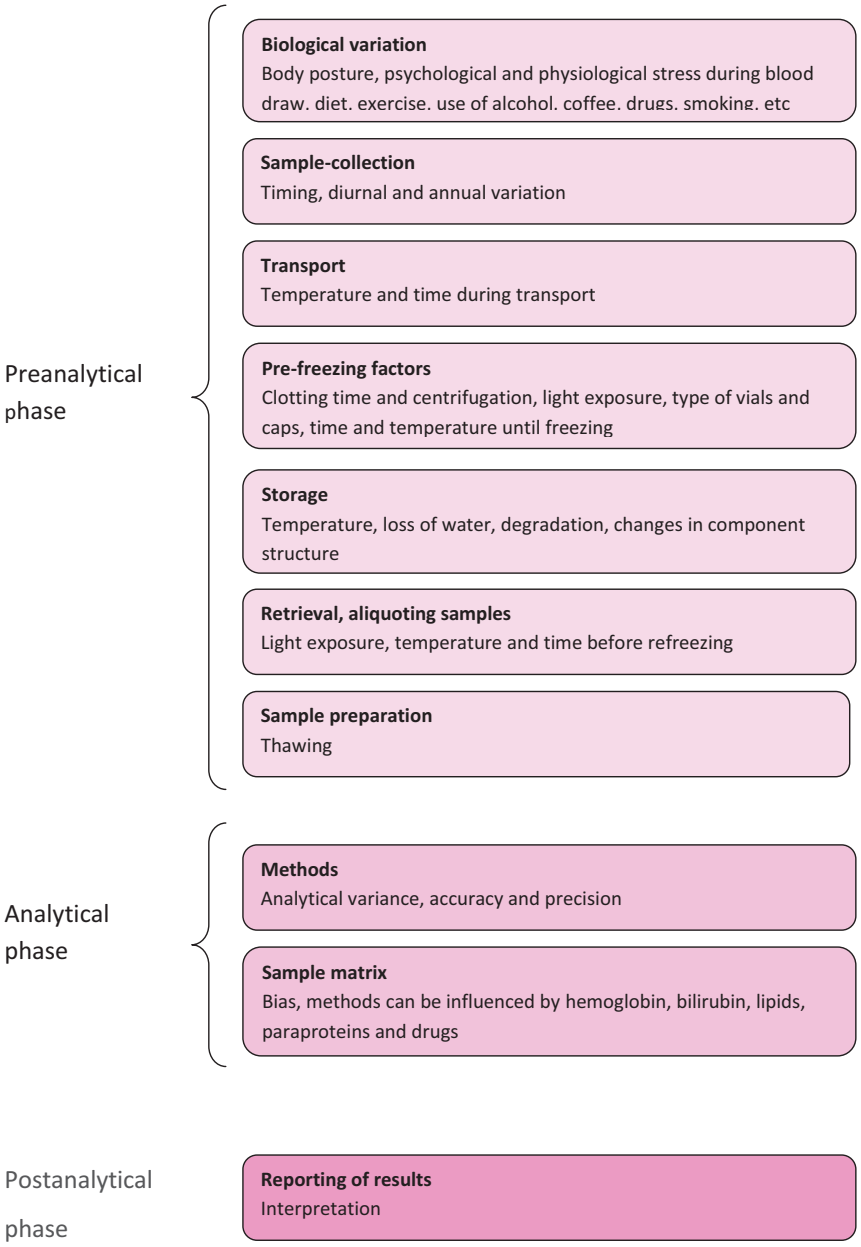

Figure 2. Factors that may affect the measurement of a biobank sample in the different analytical phases

5.6.1 Biological variation

Laboratory test results from the same individual may vary according to diurnal or biological rhythms over time. Some serum components may change from infancy throughout life, and it is necessary to stratify according to age. The level of an analyte may also have biological cycles or rhythms. Diurnal biological rhythm is well described for some hormones (*e.g.* cortisol, growth hormone, testosterone), and the monthly cycle of women in fertile age is of major importance in laboratory medicine (14). There is less documentation on seasonal cycles, although vitamin D has shown seasonal variation in serum level correlated to sun exposure (15).

5.6.2 The preanalytical phase

In general the impact of preanalytical factors is difficult to estimate due to high number of potential influencing sources. A detailed standard operation procedure (SOP) is therefore necessary to secure the quality in this phase. The preanalytical factors may be of technical or biological type. When information on technical sample handling is limited, some information may be drawn from analysing serum components known to be affected by non-optimal treatment. For instance, analysing potassium may provide information on clotting time and storage before freezing due to leakage of the component from the red blood cells (11;16). Light exposure will decrease the bilirubin concentration in a sample (11) and signs of haemolysis can indicate improper sampling or processing.

During long-term storage, the main threats against sample quality are degradation, loss of water, molecular modification, and conformational changes in protein structures. Choosing the best vials and caps for long-term storage is important. Polyethylene test tubes with screw tops are recommended to prevent desiccation(17). Sodium is an element whose serum concentration is rather insensitive to preanalytical factors and is not subjected to degradation during storage. Measurement of this element may therefore provide information on water sublimation in the vials.

Earlier studies (18-21) have addressed short-term stability at different temperatures (–20 ºC and up to +3 ºC) for different components in serum, but there are limited data for storage temperature of –25 °C and storage time exceeding more than a few years. Generally, the primary structure of proteins seems to tolerate long-term storage at –25 ºC (22) and immunoglobulins (IgE) have been proven stable at –20 ºC for up to 37 years (23;24). In contrast, proteins that are dependent of 3-dimensional structure to retain their

biological function (enzymes), a characteristic often used for measurement, are more fragile (25-27). Enzyme activity can be used as a measure of the quality of the active enzyme present. Dissociation of subunits in enzymes may decrease the activity (28). Some vitamins are very stable (vitamin A and vitamin D) for years (29;30) while Voogd et al. (31) reported substantial degradation (39%) of folate by two years of storage at –20 ºC. Thawing and refreezing may be necessary for the preparation of aliquots for laboratory analyses. It is generally regarded that fast freezing of the sera is the optimal treatment although this may not be the case for proteins. Cao et al. (32) have reported that slow freezing about 1 ºC/min and fast thawing at a rate >10 ºC/min give higher activity recovery whereas fast refreezing with slow thawing resulted in more severe damage to the proteins. Cecchini et al. (33) suggested that protein degradation is not caused by repeated freeze thaw cycles but rather by long storage in room temperature, and they therefore recommended fast refreezing.

Repeated refreezing and thawing of sera may be thought of as harmful for the sample quality. There is, however, information from a number of studies showing that several refreezing and thawing cycles do not alter assay results for many serum components. Experiments of 5–12 repeated freeze thaw cycles have shown that the serum levels remained stable for constituents like electrolytes, enzymes, protein (34), general hormones (35), female reproductive hormones (36), some biomarkers (37), and some tumour markers (34;38). However, lipoproteins and some enzymes were significantly reduced (39;40), and SHBG was reduced 3.3% per cycle (36).

5.6.3 Analytical phase

An accurate outcome of a component measurement is dependent of correct analytical procedure, including optimal instrumentation, and use of quality controls to detect errors. In this phase, errors may be identified by using strict quality criteria for the assays. The quality of the analysis can be described quantitatively by precision and trueness which refer to the results of repeated measurements. Accuracy reflects how the two former properties affect one single measurement.

Precision is the closeness of agreement between independent test results of the same sample obtained under specified condition (41). The coefficient of variation (*CV*) is a dimensionless measure of precision and is defined as the ratio of the standard deviation *s* to the mean $\overline{X}$: $CV = s/\overline{X}$

It describes the degree of random errors and is useful for comparison of data sets with different units or extensively different means. Trueness is defined as the closeness of agreement between the average value obtained from a large series of test results, and an accepted reference or consensus value. An often used indicator of trueness is bias = (mean value-true value)/(true value) (41). Bias is an expression of systematic errors. Accuracy of measurement is described as the closeness of agreement between one single measurement of a quantity and the true value of the measured component. Accuracy thus embraces the concepts of both precision and trueness, and represents the sum of random and systematic errors (total error).

Another analytical property that may affect the results is the so-called analytical specificity that refers to the ability of an assay to measure the component it claims to measure, free from interference by any other element or compound. In biochemical analysis, the matrix refers to the components of a specimen other than the analyte. The matrix may have substantial effect on the way the analysis is carried out and the quality of the results obtained; such effects are called matrix effects. There are four major endogenous matrix compounds that often interfere with laboratory analysis: haemoglobin, bilirubin, lipids and paraproteins. The major exogenous sources of interference are drugs, diagnostica, food constituents or sample additives (42).

Quality requirements

A recommended criterion for analytical imprecision is $CV_a < 1/2 \cdot CV_w$ (Cotlove's criterion) (43) where CV_a is the analytical coefficient of variation and CV_w is coefficient of variation within individual. This ensures that the variation within an individual can be monitored with an acceptable degree of precision, and the underlying data are obtained from repeated sampling from presumably healthy persons. Reference values provide a tool for interpretation of laboratory data. The reference interval embraces the total biological variance in a reference population, presumably healthy individuals, including the analytical imprecision (44). The total biological variance of a component in a given population consists of fluctuation around the homeostatic balance within the individuals (w) and between individuals or groups (g). The corresponding coefficient of variation is given by the following expression:

$$\sqrt{CV_w^2 + CV_g^2} \quad [1]$$

Including the analytical variation CV_a, the CV_{ref} for the measurements in a reference population is:

$$CV_{ref} \approx \sqrt{CV_w^2 + CV_g^2 + CV_a^2} \quad [2]$$

given that the variations are normally distributed.

Assuming good laboratory practice, analytical variance represents random errors in the method, and is for many components small compared to CV_w and CV_g. Total biological variance will therefore largely determine the reference limits. Data on the biological variability in presumably healthy individuals have been published (14;45).

A difference in mean serum component level between two populations may be found statistically significant, however not all statistically significant differences are of biologically importance. A separate criterion has been developed to evaluate the biological relevance of an observed bias (45).

An often used quality criterion for the acceptable bias is:

$$|\text{Bias}|_{\max} \leq 1/4 \cdot \text{total biological variation} = 1/4\sqrt{CV_w^2 + CV_g^2} \quad [3]$$

This criterion secures that the reference interval and hence also the localization of the study population are not substantial changed from its true value.

In situations where the separate components in [2] are unknown, an alternative formula is used:

$$|\text{Bias}|_{\max} < 1/4 \cdot CV_{ref} = 1/16 \cdot (\text{upper reference limit} - \text{lower reference limit}) \quad [4]$$

since the width of the reference interval is $2 \cdot (1.96 \cdot SD_{ref}) \approx 4\ SD_{ref}$

When comparing methods where the exact trueness of either of them is not known, an alternative bias criterion has been proposed:

$$|\text{Bias}|_{\max} < \frac{CV_w}{3} \text{ (46)} \quad [5]$$

5.6.4 Postanalytical phase

Report errors may be difficult to assess due to automation of analytical process and computer controlled systems. An irregular measurement will often be checked by a new analysis of the sample, but incorrect results within the reference interval may be accepted

more easily. In order to avoid this type of error, quality control systems should be implemented. Testing for changes from previous results (delta checks) is one possibility. Many clinical laboratories provide a fixed commentary associated with a particular test result, but correct interpretation also requires clinical data in addition of the patient's age and sex. Time of sampling and the patient status concerning diet, lifestyle, and factors that influence biological variations should also be taken in to consideration.

5.6.5 Total measurement uncertainties

Usually measurement uncertainties are considered to be a problem in the analytical phase. Kouri et al. (47) have established an uncertainty concept that take into account the patient (biological variation), the sample (handling, storage) and the analytical variation (assay). The concept is meant to assist the interpretation of laboratory results. A measurement uncertainty estimate may be important for evaluation of the confidence in a single measured value. In this work we have compared groups and described the sources of variation, but we have not numerically calculated the uncertainties of the different phases.

6. Material and methods

6.1 Material

The biological material in these studies was retrieved from the Janus Serum Bank. The samples were selected among male blood donors aged 40–49 collected in 1979, 1991, 2002, 2004, and 2008. Table 1 shows the distribution of samples, sampling years, storage time, and age in the different papers. In paper I and II the samples were analysed in 2004, while in paper III and IV the samples were analysed in 2008. All samples were retrieved, thawed in room temperature, and aliqouted before fast refreezing on dry ice**.**

Table 1. Description of material, storage time and age groups used in paper I-IV

	Paper I and II	Paper III	Paper IV
Number of Samples	390	650	520
Sampling years	1979, 2002 and 2004	1979, 1991, 2002, 2004 and 2008	1979, 1991, 2004 and 2008
Storage time	25, 2 and 0 years	29, 17, 6, 4 and 0 years	29, 17, 4 and 0 years
Age	40–49	40–49	40-49

6.2 Design

A cross-sectional design was used with groups of individuals from each year of blood draw. The groups consisted of 130 subjects of the same gender, age, and category (blood donors), and they were compared to estimate the impact of preanalytical sample handling, storage, use of different analysing methods, and potential lifestyle changes in the background population. The comparisons were based on mean value, median value, variance, and reference limits.

Ideally, for a stability study on archival serum samples, one would like to have access to identical samples retrieved from the same individuals at the same point of time, stored for different time intervals, and still have the opportunity to analyse them simultaneously with the same methods and instruments. This is clearly not possible. The two potential outlines for a stability study would then be repeated cross-sectional design and longitudinal time series design.

In the absence of historically planned longitudinal series, a cross-sectional design was chosen. Time points were selected to cover short and long-term storage. For the first study (paper I-II), we choose samples from 2004, 2002, and 1979. The analyses were performed in 2004, giving storage times of 0, 2, and 25 years. At the end of 1979, more than half of the blood donor samples in the Janus Serum Bank had been collected. This year was therefore selected as the earliest time point. In the second study (paper III), we compared 5 time points: 2008, 2004, 2002, 1991, and 1979. The analyses were performed in 2008 giving storage time of 0, 4, 6, 17, and 29 years, respectively. Folate measured by routine methods shows substantial degradation already after a few months of storage. More time points were therefore chosen to be able to assess the degrading process in larger detail. In the last study (paper IV), the time points 2008, 2004, 1991 and 1979 were selected giving storage time of 0, 4, 17 and 29 years.

The selection of individuals was random among eligible blood donors that fulfilled the requirements (sex, age, and collecting year). The blood donors constitute a subgroup in the biobank (10%) and may not be representative for all donors in the bank. Nevertheless, the use of samples from blood donors for stability studies may be justified by the fact that our question of interest was potential changes in the stored samples over time, and not the occurrence of disease in a representative population. In contrast to patient material, the blood donor group will have low prevalence of pathological laboratory results. Our age requirements were 40-49 years, and men were selected to avoid potential interference from fluctuating fertility hormones. The majority of male donors in the bank were in their forties when they donated blood, and they were therefore a suitable age group.

6.3 Biochemical analyses

In paper I, the selected components covered electrolytes (sodium, potassium and calcium), minerals (iron), and water-soluble molecules (creatinine, bilirubin and uric acid). These components were chosen to illustrate the impact of factors in preanalytical sample handling such as sublimation (sodium), clotting time (potassium), and light exposure (bilirubin). Creatinine is a marker of glomerular filtration rate (GFR) and may be used for correction of reference limits calculated for other components that may be retained when GFR decreases. The rest of the components were chosen to demonstrate component degradation or modifications, and matrix changes.

The components in paper II were selected proteins of different types and structure and included immunoglobulins, carrier proteins and enzymes (ALT, AST, CK, albumin, ferritin, transferrin, SHBG, IgE, IgG, and C-peptide). Most biomarkers investigated in the Janus Serum Bank have been proteins, and therefore knowledge of protein stability is highly relevant. Immunoglobulins have been widely analysed in cancer projects and were consequently an obvious choice. Components in paper I and II have been analysed by different assays: colorimetric, enzymatic, immuno electrochemiluminescence, immune fluorometric, immunturbidimetric, and ion selective electrode.

Paper III focused on different methods for measuring folate and folate catabolites. Some other components (homocystein, methyl malonic acid, and vitamin B12) known to reflect folate status were also measured. Impaired folate status has been associated with different forms of cancer (48), and has been used as a biomarker in epidemiological biobank studies. It has not been possible to get reliable folate measurements from archival serum samples mainly due to folate instability and methods offering low recovery. Three assays were compared 1) liquid chromatography separation followed by a tandem mass spectrometry (LC-MSMS) measuring circulating folate and oxidation products, 2) a microbiological assay measuring biological active folate, and 3) a new LC-MSMS assay using P-aminobenzoylglutamate (pABG) equivalents as a marker of total folate.

The last paper (paper IV) investigated testosterone and components connected to the testosterone level in serum (FSH, LH, albumin, and SHBG). Measured values of testosterone depend on standardization, matrix effects, and cross-reactivity to other steroid

compounds of the method used. We compared two methods: a chemiluminescence immunoassay (IMM), and a LC-MSMS method.
The serum components in paper I, II and IV were analysed at The Department of Medical Biochemistry, Rikshospitalet, Oslo University Hospital. The analyses were performed in a hospital routine laboratory using automatically analysing instruments known to minimize most of the random errors which may be of importance when studying sample stability. Most of the methods were accredited according to the Norwegian Standard NS-EN ISO/IEC 17025. The accreditation body, Norwegian Accreditation, perform technical inspection according to international rules for good laboratory practice. Testosterone was additionally analysed at St. Olav Hospital in Trondheim by a new LC-MSMS method.
The components in paper III and cotinine levels in paper IV were analysed at Bevital laboratory, Bergen. This is a commercial laboratory offering non-routine analyses. Folate was measured with three different methods. The analyses have been conducted with large automatic analysing platforms.

Samples from the different collecting time were merged and arranged in random adjacent positions in the same series to avoid bias from inter–assay variance.

6.4 Statistical analysis

To visualise the distribution form, z-score plots was used. Some of the component values were transformed (*i.e.* log or root) to achieve an approximately linear central part of the z-score plot. We applied Tukey's fence (49) for exclusion of outliers which is necessary when performing parametric tests (ANOVAs and F-test). Analysis of variance (ANOVAs) was used to identify statistical significant group differences. To test for possible heterogeneity in degradation in the groups, the variance ratio test was used. Given that there were more than two groups to compare, a Student-Neuman-Keul post hoc test was used to identify the localization of group difference. In the comparison of mean levels between the groups in the studies, the referance group (0 Year) has been regarded as the true value for the level of components. The bias criteria based on biological variation $|\text{Bias}|_{\max} \leq 1/4\sqrt{CV_w^2 + CV_g^2}$ gave the limit for what was an acceptable deviation between the compared groups. The bias criterion derived from the width of reference interval

$|\text{Bias}|_{\max} < 1/4 \cdot CV_{ref} = 1/16 \cdot$ (upper reference limit-lower reference limit) were calculated according to the RefVal method outlined in Tietz (44), and was used to control any systematic deviation from established reference limits. Comparison between methods was evaluated by a bias criterion based on the coefficient of variation within individuals $|\text{Bias}|_{\max} < \frac{CV_w}{3}$. Both the statistical and biological significances of the distribution mean difference were evaluated, the latter established with bias criterion based on biological variation in presumably healthy individuals (45). Linear regression was used to estimate the change in component concentration per year and a Deming regression was used to compare two methods. A significance level of 5% was chosen except for the F-tests in paper III were Bonferroni correction yielded significance level of 5%/3=1.7%.

7. Brief summary of results

7.1 Paper I

Long-term stability of serum components in the Janus Serum Bank

In order to investigate preanalytical sample handling we assessed the long-term (25 years) and short-term (2 years) stability of seven biological components in serum samples stored at –25 °C. Specimens from male donors, 130 in each group, stored for 25 and 2 years, were compared with 130 one-month-old samples. The distributions, dispersions, medians, means, and established reference intervals were used for comparisons. A significant difference in sodium of +3.9% in long-term stored serum samples demonstrated a small degree of sublimation during storage. In the 2 year-old samples an increased level of potassium (+19.9%) was interpreted as the influence of prolonged clotting time. The altered level of bilirubin (–32.4%) illustrated the effect of light exposure during preanalytical sample-handling. The differences for calcium and iron which should be as inert substance as sodium, did not reach statistically significance probably due to greater variation (analytical and biological).

7.2 Paper II

Stability of selected serum proteins after long-term storage in the Janus Serum Bank

The stability of selected proteins; immunoglobulins, carrier proteins and enzymes in samples stored at –25 °C were investigated. Three groups of 130 male samples stored for 25 years (long-term), 2 years (short-term), and 1-month-old samples, respectively, were compared. The distribution of the values was examined, dispersion and localization of central tendency were compared, and reference intervals for each component were established. Albumin, AST, cystatin C, IgE, IgG, SHBG, and transferrin showed non-significant or numerically small group differences. Large differences after 2 years of storage was seen for ferritin (–18.5%), ALT (–41.1%), CK (–41.1%), while insulin C-peptide nearly totally decayed after long-term storage (–98.7%). The study demonstrated that immunoglobulins, which are often analysed in the Janus Serum Bank, are relatively robust to long-term storage. Enzymes, where alteration in the 3-dimensional structure or modifications that affect the catalytic site of the enzyme, may be critical for their properties and function, appeared to be more fragile.

7.3 Paper III

Analytical Recovery of Degraded Folate in Archival Serum Samples

Folate status is often addressed in association with cancer. Measurement of folates in archival serum samples has proved difficult due to degradation during storage. Analyses that include degradation products may improve the results compared to routine immunological methods. We determined folate in human serum stored at –25 °C for 0, 4, 6, 17, and 29 years by three different folate assays: 1) a microbiological assay, 2) a LC-MSMS assay that measures different folate species, including 5-methyltetrahydrofolate (5mTHF) and the oxidation product 4-alpha-hydroxy-5-methyltetrahydrofolate (hmTHF), and 3) an assay that converts folate species to p-aminobenzoylglutamate (pABG), which is then quantified by LC-MSMS. Although the loss of folate measured by the folate LC-MSMS assay was less than for the microbiological assay, it was still substantial. The study showed a recovery of 41.3, 55.7 and 78.9% for folate determined by microbiological assay, LC-MSMS assay and as pABG equivalents, respectively. For assessment of the anticipated initial level, folate determined as pABG equivalents gave the most reliable result.

7.4 Paper IV

Stability of testosterone level in archival serum samples – methodological approaches, and changes in the background population

The association between low hormone levels and impaired health status like the metabolic syndrome and cancer make measurement of testosterone important in epidemiological studies. Standardization, matrix effects, and cross-reactivity to other steroid compounds are of importance for the method used. Testosterone was measured by a chemiluminescence immunoassay (IMM), and a LC-MSMS method. A Deming regression gave comparable results for both methods for testosterone levels in the range 5–30 nmol/L. We found a substantial higher mean level of testosterone (ANOVAs, $p<0.0001$), 28.2% and 24.4% for IMM and LC-MSMS methods, respectively, in the oldest group (29 years) compared to the 0 year group. The study demonstrated no impact of age, season or smoking. The higher mean levels of testosterone in the oldest groups indicated no degradation during storage, and the lower level in the fresh samples could suggest changes

in the background population during a time span of 29 years (1979 – 2008) among the men investigated in this study.

Table 2. gives an overview of the studies, including the main results.

Table 2. Overview of the material, statistical analyses, and main results from the studies

	Paper I	Paper II	Paper III	Paper IV
Aims	To investigate the impact of preanalytical handling and storage condition	To test the long-term stability of biomarkers	To estimate folate recovery	To investigate the impact of storage, method and changes in background population on testosterone level
Study design	Cross- sectional	Cross- sectional	Cross- sectional	Cross- sectional
Study population	Men, 40-49 years Red Cross Blood Bank donors	Men, 40-49 years Red Cross Blood Bank donors	Men, 40-49 years Red Cross Blood Bank donors	Men, 40-49 years Red Cross Blood Bank donors
No of person and samples included	3 groups of 130 individuals Total: 390 samples	3 groups of 130 individuals Total: 390 samples	6 groups of 130 individuals Total: 780 samples	4 groups of 130 individuals Total: 520 samples
Year of blood draw	1979, 2002, 2004 Analysed in 2004	1979, 2002, 2004 Analysed in 2004	1979, 1991, 2002, 2004, 2008 Analysed in 2008	1979, 1991, 2004, 2008 Analysed in 2008
Statistic	z-score plot , Tukey's fence, ANOVAs, Student-Newman-Keul, REFVAL, F-test, and biological based bias criterion	z-score plot , Tukey's fence, ANOVAs, Student-Newman-Keul, REFVAL, F-test, and biological based bias criterion	z-score plot, Tukey's fence, Linear regression, F-test, and biological based bias criterion	z-score plot, Tukey's fence, Deming regression ANOVAs, Student-Newman-Keul, REFVAL, F-test, and biological based bias criterion for comparison of component levels and methods
Main results	Differences between groups indicated influence of delayed clotting time, light exposure and loss of water during storage	Immunoglobulins are relatively stable, some enzymes were particularly fragile	New method give better recovery (≈ 80%) of folate in archival serum samples	An immunological and a LC-MSMS method gave similar results for testosterone level. Substantial lower level of testosterone in fresh samples compared to 29 years old ones, suggested changes in the background population

8. General discussion

The present work has addressed five main issues relevant to examination of archival serum samples: 1) differences induced by non-standardised preanalytical sample handling, 2) sublimation during storage, 3) component stability, 4) recovery of a degraded serum component (folate and folate derivatives) by a new assay, and 5) possible time trends in population levels of serum components. The stability studies have measured a number of components *i.e.* electrolytes, metabolites, enzymes, immunoglobulines, transport proteins, vitamins and hormones. The main results in paper I – III showed small or no changes in serum level for calcium, iron, creatinine, uric acid, albumin, AST, cystatin C, IgE, IgG, SHBG, and transferrin after 25 years of storage at –25 ºC. On the other hand, ferritin, ALT, CK, folate and folate-deratives were subjected to degradation, and the choice of analysing methods may be essential for the possibility to assess the original level of the component. For some enzymes the technique of measuring activity in archival samples should be replaced by measurements based on other characteristics *e.g.* mass determination by immunochemical techniques. Paper I examined some components (bilirubin and potassium) that illustrate some of the factors that contribute to preanalytical errors of which the information often is scarce. Increased component level due to water sublimation during storage can be revealed by measurement of sodium in a group of samples from different time points. Without this correction, small differences in component level may be masked or exaggerated. In paper IV we found the highest level of testosterone in the oldest samples which indicated a decreasing trend in the background population that may have masked a possible degradation. Besides the process of degradation there are three main topics to be discussed as to the validity of these results: the impact of sample handling, the study design in the present studies, and possible changes in the background population over time.

8.1 Sample handling

The Red Cross Blood Bank had collecting procedures that ensured a relatively similar sample handling over the years. Only the storage time of centrifugated whole blood samples (clotting time) before separation of the serum fraction may have differed, varying between 14 and 28 hours. The lack of specific information on each sample is a weakness. To estimate the potential contribution from differences in clotting time on proteins, an experiment was performed and reported in paper II. Only minor changes were found for C-peptide, SHBG,

ferritin, ALT, AST, and CK with time, indicating that the delayed clotting times of 14, 22, and 28 hours had negligible impact on the serum proteins investigated. In paper III two paralleled 2008 samples were collected to investigate the impact of delayed clotting time on the folate metabolites. There was no statistically significant difference in folate concentration between the two parallels, which indicated that a difference in 6 hour of clotting time would not seriously affect the level of the different folate markers (data not published).

The majority of the samples in the Janus Serum Bank has been collected during health examination surveys across the country and were subjected to delay in freezing of the serum fraction due to long-distance transport. Reduced quality may have occurred although the samples were transported on a cooling system that ensured a temperature between +3 ºC and +10ºC (2). Other studies, however, report no significant changes in level of common biological analytes (i.e. proteins and electrolytes) in rat serum under refrigeration for 7 days (50), and (*i.e.* cholesterol and enzymes) in human serum under refrigeration for 4 days (50;51).

The observed differences in component levels over time may therefore have been caused by storage related changes. Degradation, conformational changes, molecular modifications or water sublimation may occur. The measuring methods can introduce errors that may appear as stability problems even in relatively fresh sample (52). Methods based on measurements of enzyme activity and the use of immunological assays with monoclonal antibodies, are especially vulnerable in this respect. In paper I and II we analysed our samples with routine instruments at a clinical hospital laboratory. The instruments had stable *CVs* over time and were run under strict quality assurance.

The effect of the freeze and thaw procedures and repeated freeze and thaw cycles has not been addressed in the present thesis. In the Janus Serum Bank all samples retrieved for projects have to be thawed and refrozen at least once for aliquoting, and the procedure is to thaw slow at room temperature and freeze fast on dry ice. There are contradictory reports in the literature on the impact on antibodies, electrolytes, glucose, and enzymes (33;34;36-39;48) but we may assume that any influence of the freezing and thawing procedure would affect all samples in the same way.

8.2 Study design

A cross-sectional design provides a snapshot of the outcome of interest at a particular point of time. When several cross-sectional sample collections are compared, people with some similar background characteristics are grouped although none is followed individually (53). In our design it may be difficult to distinguish between inter-individual differences and difference caused by storage. To overcome this, the sample size at each time point has to be large and sampled randomly from the population. To establish reference limits with confidence intervals it is recommended to use at least 120 subjects (44;54). We included 130 individuals in each group to meet this requirement and still had the option to remove obvious outliers. We have found differences over time for several components. The questions arise whether these differences were statistically or clinically significant, and if the sample size was large enough. The interpretations are based on adequate statistical methods and on well established bias criteria. The number of individuals in each group is large enough to assure a proper determination of reference limits, but still there might be type I and II errors when comparing component levels.

In the Janus Serum Bank blood donors have given samples repeatedly. Longitudinal time series was therefore an option that was discussed, the advantage being the possibility to follow the same person and describe the pattern of changes over time. Repeated observations at the individual level may offer an advantage over a cross-sectional design, as it may eliminate inter-individual biological variation, when an individual serves as his or her own control. The drawback using the longitudinal time series design may be the difficulties to obtain sufficiently large number of individuals with samples collected over large enough time span and at similar intervals. This type of study would still be affected by potential changes with increasing age (*e.g*. decreased hormone levels), and thereby confound the comparisons between samples with different storage times. Longitudinal sampling from the same individuals may also suffer from unregistered changes in diet, infections, or seasonal and cyclic variation.

The use of samples from males only, may imply that some results are not representative for female donors. Males were chosen for all studies because they have less intra- and inter-individual biological variation linked to sex hormones. Concerning the aspects of component stability in a biobank, it is unlikely that great difference between gender should exist.

The selection of components does not cover every aspect of quality in a biobank, but represented a broad spectrum of structurally different molecular component, many of them relevant to cancer research. However, it is impossible to generalise between components. Analogous components may decompose differently. In paper II we found that the two liver enzymes AST and ALT behaved very differently under storage; AST was relatively stable while ALT lost 73.4% of its activity after 25 years of storage.

8.3 Lifestyle related changes in the background population

A historical biobank will also reflect time specific changes in the background population, and differences between old and new samples may therefore be caused by other than storage related factors. In paper IV we found a substantial higher testosterone level in the oldest samples that could not be explained by factors like age, diurnal or seasonal variations. Storage-related changes like degradation could have occurred, but are not very likely. The real difference in testosterone level would then have been even larger. Higher serum testosterone levels have been associated with smoking (55;56). Cotinine, the main metabolite of nicotine is believed to be a very stable compound (57), and can therefore serve as a measure of tobacco exposure (58;59). Figure 3 shows the results of cotinine measurement in serum from four groups of male donors in the Janus Serum Bank collected in 1979, 1991, 2004, and 2008. The difference in smoking pattern is in agreement with the change seen in the general Norwegian population (60). In the period 1972–2006 the proportion of male daily smokers in Norway was reduced from 61% to 27% (61). For men aged 45–54 years the proportion in 1979 and 2008, was 46% and 26%, respectively, and corresponding for Janus 38.2% and 30%.

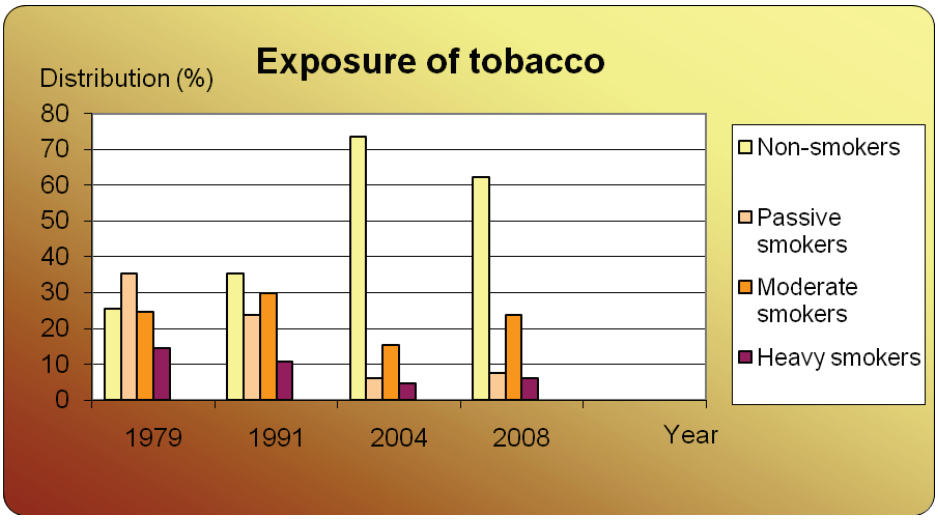

Figure 3. Exposure of tobacco in males from 4 collecting times in the Janus Serum Bank

The prevalence of smoking has been reduced over the years, but we found no statistically significant correlation between cotinine and testosterone in our material. A number of acute and chronic changes in serum component concentrations, the chronic being relatively modest, are associated with smoking. Constant smoking may induce increased blood glucose, changes in leukocyte and erythrocyte counts, lipoproteins, activity of enzyme (angiotensin converting enzyme), hormone (prolactin), vitamins (pyridoxal phosphate, β-cartinoids), tumour marker (CEA), and heavy metals (copper, lead, cadmium and selenium) (62). Albumin was reported to increase by 3% in smokers. A correction of albumin levels in all groups according to cotinine levels may have increased the + 4.8% difference between the 1979 and 2008 samples (paper II)

In the period 1973–2004 the alcohol consumption has changed in the Norwegian population by an increase in number of persons who consume alcohol relatively frequently, and the proportion that drink more than 10L pure ethanol per year has doubled (63). The chronic effects of alcohol abuse include increased serum activity of liver enzymes and triglycerides (62). The aminotransferases ALT and AST activities are increased due to direct liver toxic effects and was reported changed by 50% and 200%, respectively. The selection of individuals (blood donors) in our study, weigh against a high number of alcohol abusers, and

we have concluded that the trend of increased alcohol intake would not be expected to influence our results.

Some other relatively large changes in lifestyle and socio-economic standards have also taken place in the Norwegian population during the last three decades. The most remarkable one is the prevalence of obesity. In the period 1963–95, the mean weight increased 5.6 kg for men and 1.3 kg for women. Body mass index (BMI) has increased steadily in men and the proportion with BMI above 30 kg/m^2 was doubled during the period (64). A comparison study among men in Oslo aged 20-49 years between the time points 1972 and 2000, showed a change in mean BMI from 24.3 to 26.4 kg/m^2 (65). We have no information on BMI for the blood donors, but we assume that the general change in BMI also may have affected the participants in our studies. Increased BMI is associated with reduced testosterone level (66;67) and may have contributed to the lower testosterone level in the 2008 group.

Norwegian diet during the first decade after the Second World War was characterized by a high consumption of fat. In the period 1975–2000 the fat intake was reduced from 40 to 35 energy percent (68). A drop in serum cholesterol level of 0.6–0.8 mmol/L (8.0–11.4%) in the Norwegian population between 1974 and 2002 has been demonstrated (69). Although cholesterol is a precursor of steroid hormones, the regulation of steroids are assumed to be largely independent of plasma cholesterol levels within the reference range. Dietary data for this period (70) show that the intake of vegetables, fruits, and juice has increased considerably and thus increased the consumption of vitamin C. Recently, vitamin and mineral enrichment of food in Norway has become allowed for several products, which might further increase the circulating level of these components. The use of dietary supplements (vitamins) is increasing in Sweden and Denmark (71;72), and probably in Norway as well, although exact data is lacking. We have investigated folate levels during the time period of 1979–2008. Folate degrades rapidly in serum and its instability makes it impossible to estimate any effect of increased intake of folate supplements.

The alteration in the background population should be taken in to account when measuring components that may have been influenced by such changes.

9. Concluding remarks

The quality of the samples is a very important aspect in all research projects that uses archival material. The aim in this work has been to investigate to what extent the samples in the Janus Serum Bank, reflect the biological, or biochemical situation in the individual at the time of sample collection. Different factors that may influence on the concentration of a sample component during collection, storage, and analysing have been discussed.

Based on the results of this thesis it can be concluded:

- Long-term stored serum samples seem to undergo a modest water sublimation that can be corrected for in research projects.
- Some information on the variation of preanalytical sample handling can be assessed by analysing components that are influenced by clotting time, light exposure and haemolysis. In the absence of standardised sample handling, individual measurements of unstable parameters should be interpreted with caution.
- Many proteins, *e.g*. immunoglobulins, that are often analysed in the Janus Serum Bank, are robust to long-term storage at –25 °C.
- Choice of measurement technology may strongly influence the results for components that tend to degrade.
- Changes in lifestyle over time in the background population may introduce confounding or bias in studies of archival biobank material.

Despite a sub-optimal preanalytical sample-handling and a higher storage temperature compared to modern biobanks, the present thesis shows that the Janus serum samples are of adequate quality for a number of purposes. The combination of knowledge of component stability, an adequate design, and use of an optimal assay is essential.

When examining components with unknown stability one is advised to run pilot studies to measure the levels of these in samples with different storage time and explore their distributions. This work advocates for the routine of proper matching of cases and controls according to time of blood draw, to minimize the impact of preanalytical handling, storage, and lifestyle effects.

10. References

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